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# A novel *Leishmania infantum* nuclear phosphoprotein Lepp12 which stimulates IL1-beta synthesis in THP-1 transfectants

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## Abstract

**Background:** We report cloning and characterization of a novel *Leishmania infantum* protein which we termed Lepp12, and we examine its possible implication in the interference with intramacrophage signaling pathways.

**Results:** The protein Lepp12 contains 87 amino acid sequence and exhibits 5 potential phosphorylation sites by protein kinase C (PKC). Recombinant GST-Lepp12 is phosphorylated *in vitro* by exogenous PKC and by PKC-like activities present in promastigote and in the myelomonocytic THP-1 cell line, indicating that at least one phosphorylation site is functional on the recombinant Lepp12. The natural Lepp12 protein is present in *L. infantum* promastigotes, as evidenced using specific anti-Lepp12 antibodies produced by immunopurification from acute phase VL patient sera. Interestingly, human patient sera are strongly reactive with GST-Lepp12, demonstrating immunogenic properties of Lepp12 in man, but no immune response to Lepp12 is detectable in experimentally infected animals. When isolated from promastigotes, Lepp12 migrates as two species of apparent MW of 18.3 kDa (major) and 14 kDa (minor), localizes in the nuclear fraction and appears constitutively phosphorylated. Natural Lepp12 is phosphorylable *in vitro* by both exogenous PKC and PKC-like activity present in THP-1 extracts. The intracellular Lepp12 transfected into THP-1 cells activates these cells to produce IL-1beta and induces an enhancing effect on PMA stimulated IL-1beta synthesis, as demonstrated using GST-Lepp12 transfectants.

**Conclusions:** Together these results indicate that Lepp12 represents a substrate for PKC or other PKC-like activities present in the promastigote form and the host cell and therefore may interfere with signal transduction pathways involving PKC.

## Background

Leishmaniases are parasitic diseases due to protozoa of the genus *Leishmania* transmitted by sandflies of the genus *Phlebotomus*. In the vertebrate host,*Leishmania* live in macrophages as obligate intracellular amastigotes, and as flagellated free promastigotes in the intestine of the sandfly vector. There are at least 20 different species of *Leishmania* parasites causing a wide spectrum of human diseases, ranging in severity from spontaneously healing skin lesions to fatal visceral leishmaniasis [1,2]. The prevalence of the disease worldwide is estimated to be 12 million cases and an incidence of 500 000 new cases of visceral and 1 500 000 of cutaneous disease has been reported [1]. Patent visceral leishmaniasis (VL) caused by *L. infantum* (*L. chagasi*) is a fatal infection when left untreated [2]. There is an increasing incidence of the disease in HIV-infected individuals in southern Europe, [3,4] and post-therapeutically, in organ transplantation [5]. This is due, in part, to the reactivation of latent *Leishmania* in persons presenting immunosuppressed conditions [4]. Indeed, in endemic regions the existence of asymptomatic *Leishmania* carriers has been documented [6,7] and in successfully treated VL patients the currently available drugs do not result in the complete elimination of the parasite.

*Leishmania* parasites developed various strategies to overcome the protection provided by the immune system of the host [for review [8,9]]. In particular, phosphorylation reactions have been shown to participate in several ways in escape mechanisms, at different levels of the parasite-host interaction. For instance, a protein kinase isolated from *L. major* (LPK-1) is able to phosphorylate components of the human complement system (C3, C5 and C9) leading to its inactivation [10]. Intracellular *Leishmani*a amastigotes, not only adapt to phagolysosomal low pH (5.5) and high temperature (37°C) in order to survive in the host cells [11,12], but also induce functional modifications in macrophages. These include decrease in cytokine production, inhibition of oxydative burst activity, alteration of antigen presentation, and of expression of MHC class II molecules. This ability of *Leishmani*a to inhibit macrophage effector activities, also termed deactivation [8,13], may result from a direct interference of leishmanial molecules with macrophage signal transduction pathways. In particular, inhibition of macrophage protein kinases such as protein kinase C (PKC) [14–16] and Janus kinases [17,18], as well as alteration of stimulus-induced intracellular calcium gradient and decreased production of inositol 1, 4, 5-triphosphate [19,20] have been reported. The inhibition of PKC-depending signaling by *Leishmania* is well documented, and the effect can be ascribed in part to the properties of lipophosphoglycan (LPG) [21–28].

In this paper we report cloning and characterization of a novel *L. infantum* protein termed Lepp12, the predicted aminoacid sequence of which contains 5 potential sites of phosphorylation by PKC and examine its possible implication in the interference of intramacrophage signaling pathways.

| | |
|---|---|
| G**KRT**KKAPKQKRKLAMADRPR **KLT**SKGKV | 29 |
| KHKRGDLKMVSS **RNVS**FDVRQGKGGKWIK | 58 |
| TGERKCNIHTVCDCV **KRT**DPARMRYI **TNR** | 87 |

**Figure 1**
Predicted amino acid sequence of Lepp12. Motives corresponding to potential phosphorylation sites (bold), and potential N-glycosylation site (underlined) are given by the consensus sequences found by the BLAST search method. cDNA sequence is available under the accession number AF540954 in GenBank.

## Results

### *Cloning of a novel* L. infantum *cDNA and production of recombinant protein GST-Lepp12*

After screening two expression *L. infantum* cDNA libraries with an acute-phase VL patient serum a 214-bp lambda gt11 insert was selected and sequenced of as described previously [29]. Then, the 267-bp ORF of Lepp12 was obtained using RACE-PCR on retrotranscribed promastigote mRNA, as indicated in the Methods section [29]. Figure 1 shows the deduced 87 amino acid sequence of predicted molecular weight of 11.6 kDa. Its analysis exhibits 5 potential phosphorylation sites (bold characters) and one N-glycosylation site (underlined). No homologies of Lepp12 with sequences of *Leishmania* proteins reported to date were found. The recombinant GST-Lepp12 protein migrates, as expected, at 38.5 kDa (Figure 2, lane 1). It was produced in parallel with GST (Figure 2, lane 2), as a control. The very faint band at 34 kDa could correspond to a proteolytic degradation of the fusion protein during its production by *E. coli* bacteria, according to a recently published study [30]. A yield of approximately 30 microg of GST-Lepp12 per 100 ml culture was determined after electrophoresis gel staining.

### *Identification and localization of the natural Lepp12 in* L. infantum

In order to evidence a corresponding natural Lepp12 in *L. infantum* promastigotes, specific anti-Lepp12 antibodies were first isolated. Interestingly, no immune response to Lepp12 was detected in experimentally infected animals ($10^7$ stationary promastigotes per animal by iv and ip route for mice and hamsters, respectively; not shown), while immunization of hamsters with GST-Lepp12 resulted in sera presenting a quite low titer of anti-Lepp12 antibodies (not shown). Conversely, as shown on Figure 3 sera collected from patients at VL diagnosis are strongly reactive with the fusion protein GST-Lepp12, demonstrating immunogenic properties of Lepp12 in man. Of note,

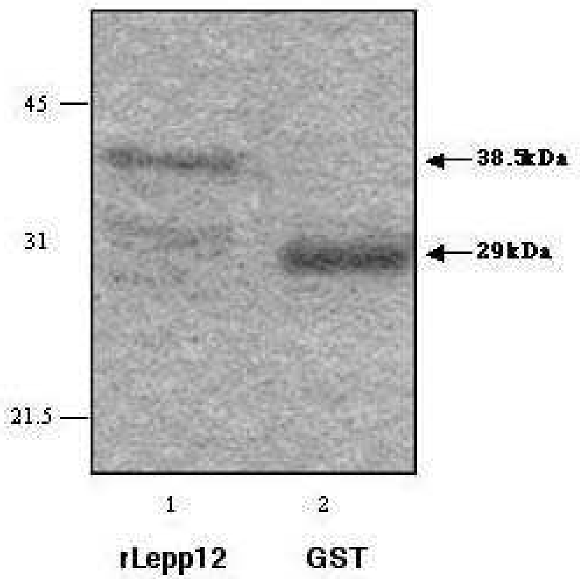


**Figure 2**
Purification of recombinant proteins. The recombinant proteins GST-Lepp12 (rLepp12, 38.5 kDa, lane 1) and GST (29 kDa, lane 2) were purified from *E. coli* BL21 induced cultures (0.1 mM IPTG for 2 h). Bacteria were extracted with 1% Triton X-100 and fusion proteins were purified on gluthatione sepharose column. Five microg of eluted material was analyzed by SDS-PAGE (14% polyacrylamide) and Coomassie Blue staining. Molecular mass markers are indicated in kDa.

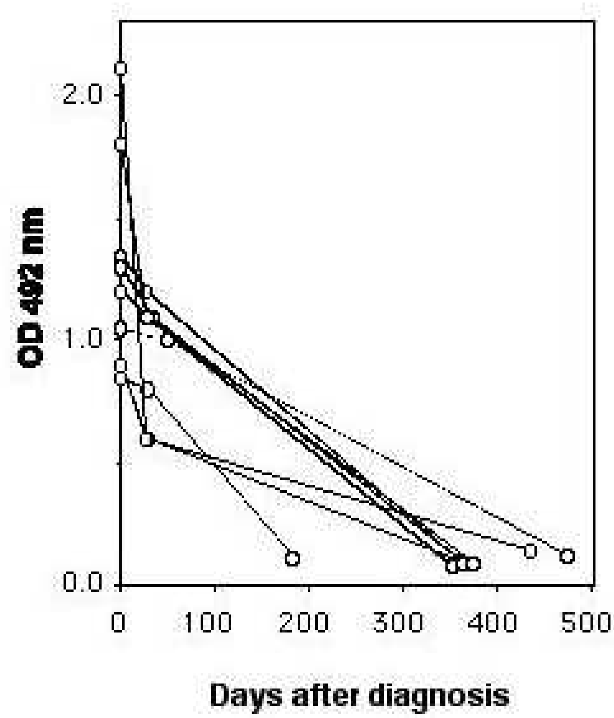


**Figure 3**
Anti-Lepp12 antibody responses in patients with clinically and parasitologically diagnosed VL. Reactivities of diagnosis and follow up sera against recombinant Lepp12 from eight VL patients are shown. GST-Lepp12 and GST, purified as described in Methods section, were eluted from gluthatione sepharose with 0.1% SDS in PBS. Recombinant proteins were coated at 0.05 microg per well onto microtiter plate and sequentially incubated with patient serum diluted 1:100 and peroxidase-labeled anti-human IgG. Absorbencies obtained with GST-Lepp12 were corrected for absorbencies measured with GST alone. Sera from leishmanin skin test (LST) negative controls gave OD values below 0.1. The patient's serum with the highest titer at diagnosis was selected for the preparation of the immunopurified anti-Lepp12 antibodies. Typically 20 microg of immunopurified anti-Lepp12 antibody were obtained from 1 ml of serum.

sera from LST positive asymptomatic subjects were not reactive (not shown). Therefore to obtain the specific anti-Lepp12 IgG we performed immunopurification of sera from patients presenting strong anti-GST-Lepp12 responses, as described in the Methods section.

The natural Lepp12 protein in *L. infantum* promastigotes was then identified by western blotting. It migrates as one intense immunoreactive band at 18.3 kDa and one weaker band at 14 kDa, (Fig. 4 lane 4). The reactivity of the used immunopurified antibody on the recombinant GST-Lepp12 and on GST and GST-papLe22 [29] are shown as positive (lane 1) and negative (Fig. 4, lanes 2 and 3) controls, respectively. At first sight it might appear surprising that, as evidenced in Fig. 4, 5 microg of recombinant protein Lepp12 are recognized with almost the same intensity as the natural Lepp12 protein contained in 15 microg of the promastigote lysate. But one has to remember that the source of antibodies used in these experiments were human patients sera and therefore the antibodies, although affinity purified on rLepp12, are originally directed against the natural Lepp12. The expression of Lepp12 cDNA in *L. infantum* promastigotes and amastigotes was demonstrated by RT-PCR (not shown). In order to determine the natural Lepp12 localization in cellular compartments, western blotting was carried out after promastigote fractioning, using immunopurified anti-Lepp12 antibodies. Figure 5 reveals the same two immunoreactive bands, the intense at 18.3 kDa and the weak at 14 kDa in the total unfractionated promastigote SDS lysate (Fig. 5 lane 1), and in the nuclear extract (Fig. 5 lanes 5 and 6), and no detectable material in the remaining cell fractions. These

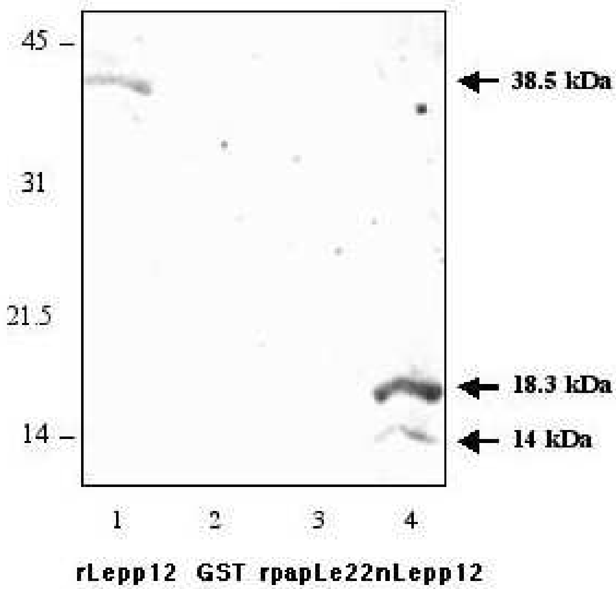


**Figure 4**
Identification of natural Lepp12 (nLepp12) in promastigotes. Five micrograms of GST-Lepp12 (rLepp12, lane 1) used as positive control, or GST (lane 2) or GST-papLe22 (rpapLe22, lane 3) used as negative controls, or 15 microg of leishmanial proteins (total promastigote lysate in SDS 3%, lane 4) were electrophorezed and electrotransfered onto nitrocellulose. Western blotting was performed with anti-Lepp12 antibodies (at 2 microg/ml) immunopurified on Lepp12-coated latex beads from an acute phase VL patient serum, which was previously absorbed with 0.5 ml of *E. coli* lysate. Molecular mass markers are indicated in kDa.

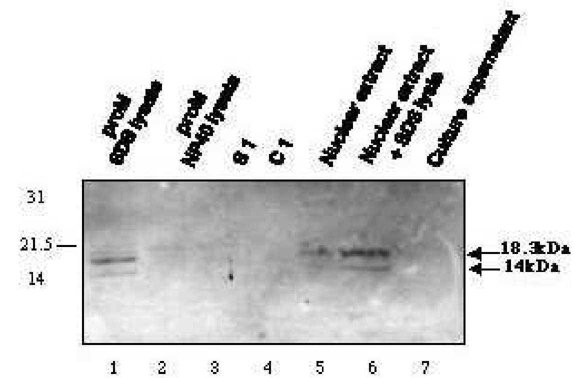


**Figure 5**
Natural Lepp12 protein is non-secreted protein located in promastigote nucleus. Western blotting was performed using immunopurified anti-Lepp12 antibodies (at 2 microg/ml) on total promastigote 3 % SDS lysate (positive control, lane 1), 20 % NP40 lysate (lane 2), cytosol (s1, lane 3), cell membranes (c1, lane 4), nuclear extract prepared as described in Methods section before (lane 5) or after (lane 6) 3% SDS lysis, and PEG concentrated culture supernatant (lane 7). Proteins were prepared from $1.6 \times 10^8$ cells. In each lane, 30 microg of proteins were separated on a 14% polyacrylamide SDS-gel, corresponding to an equivalent of 4 millions (lane 1), 8.6 millions (lane 2), 5.3 millions (lane 3), 4.8 millions (lane 4), 18.5 millions (lane 5), 12 millions (lane 6) and 2.2 millions (lane 7) promastigotes. Molecular mass markers are indicated in kDa.

results indicate that natural protein Lepp12 is located in promastigote nucleus. The presence in the Lepp12 amino acid sequence of a motif associating a proline with 3 basic amino acids (Figure 1, RPKR, aa 19 to 22), might be indicative of the nuclear location of the protein.

### In vitro *phosphorylation of recombinant Lepp12*
Several *in vitro* assays were carried out in order to examine the capacity of Lepp12 to get phosphorylated. In a first series of experiments the recombinant protein was incubated with purified exogenous protein kinase C (PKC). Figure 6 shows a phosphorylated band migrating, at the MW of 38.5 kDa (lane 4) corresponding to GST-Lepp12. Preparations of the GST protein (lane 6), of a lysate of not transformed *E. coli* (lane 5), both produced in parallel with GST-Lepp12, and reaction mix alone (lane 2) were also incubated with PKC to provide negative controls. A histone mix was used as a positive control of phosphorylation reaction (lane 1). This result indicates that among the potential phosphorylation sites of Lepp12, al least one site is functional and that recombinant Lepp12 is a substrate of PKC *in vitro*. Next, the promastigote lysate, added to the recombinant Lepp12, was used as a possible source of enzymatic activity in an *in vitro* assay. Figure 7 shows that, indeed, a kinase activity able to phosphorylate GST-Lepp12 (lane 7), and also the histone mix (lane 3), is present in promastigotes. In this experiment, phosphorylation of the fusion protein GST-Lepp12 by the exogenous PKC was used as positive control (lane 5). The enzymatic activity present in promastigote lysate was inhibitable by kinase inhibitor bisindolylmaleimide VIII (data not shown) when it was used at the concentration of 1 microM, suggesting its PKC-like character.

### *Phosphorylation of natural Lepp12 protein*
First, we checked that natural Lepp12 was phosphorylated by exogenous PKC in an *in vitro* phosphorylation assay (not shown). Then, in order to find out what is the phosphorylation status of the natural Lepp12 in promastigotes, the protein was first immunoprecipitated from the nuclear extract using specific immunopurified anti-

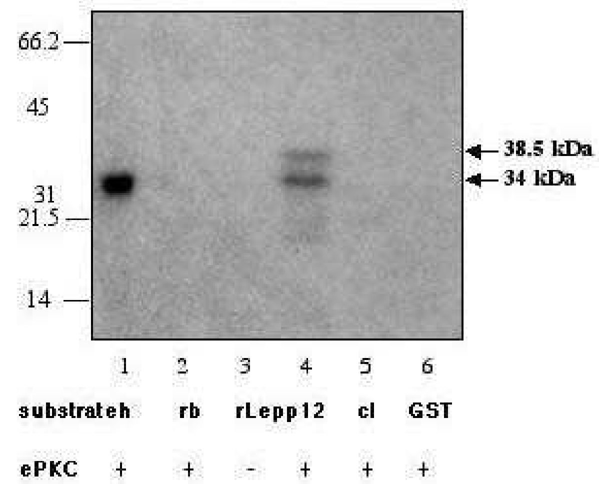


**Figure 6**
Recombinant protein Lepp12 is phosphorylated by purified exogenous PKC (ePKC) in the presence of 10 microCi gamma-$P^{32}$ ATP in an *in vitro* assay. rLepp12: GST-Lepp12; h: histone mix as positive phosphorylation reaction control; GST; rb: reaction buffer; cl: *E. coli* lysate, as negative controls. Autoradiogram is shown after exposition time of 30 min.

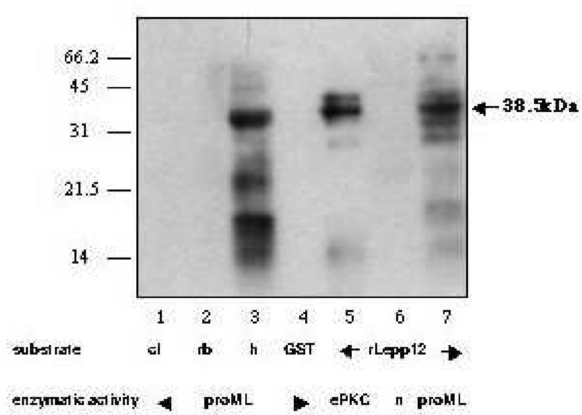


**Figure 7**
Recombinant protein Lepp12 is phosphorylated by an enzymatic activity present in promastigote lysate (proML), in the presence of 10 microCi gamma-$P^{32}$ ATP in an *in vitro* assay. rLepp12: GST-Lepp12; h: histone mix as positive phosphorylation reaction control; GST; rb: reaction buffer; cl: *E. coli* lysate, as negative controls. n = no enzymatic activity added. Autoradiogram is shown after exposition time of 24 hours.

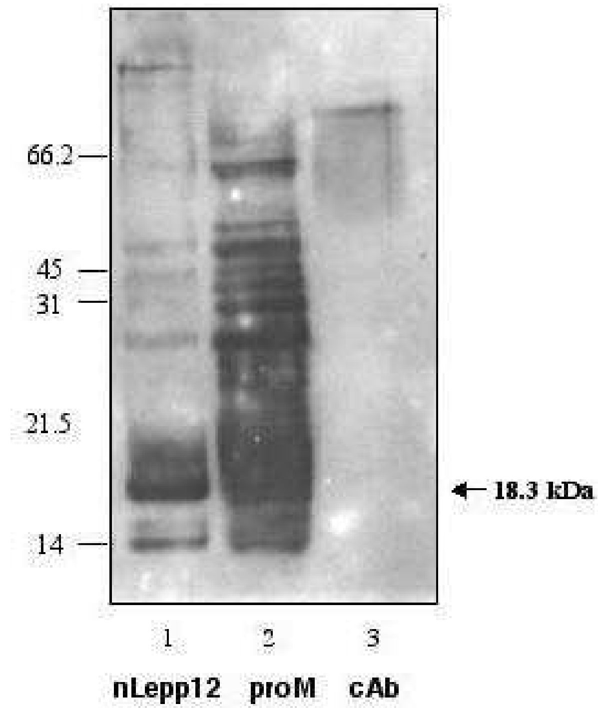


**Figure 8**
Natural Lepp12 is present in promastigote nucleus as a phosphoprotein. Mixture of anti-phosphoserine and anti-phosphothreonine antibodies was used in the western blotting shown in order to evidence phosphoproteins. Enzymatic activity of anti-rabbit IgG peroxidase conjugate was revealed by chemiluminescence. Natural Lepp12 (nLepp12, lane 1) was immunoprecipitated from the promastigote nuclear extracts (corresponding to $3 \times 10^9$ cells) using anti-Lepp12 IgG (25 microg at 100 microg/ml). The immunoprecipitated material was Lepp12-specific as testified by control immunoprecipitation (cAb) performed with irrelevant IgG (lane 3). 15 microg of total promastigote lysate (proM, lane 2) was electrophorezed and electrotransfered onto nitrocellulose in parallel. Exposition time of MP-hyperfilm was 10 sec.

Lepp12 antibodies as described above, and western blot analysis was next carried out using a mix of anti-phosphoserine and anti-phosphothreonine antibodies. Figure 8 shows that the natural Lepp12 immunoprecipitated from the nuclear extract was revealed by anti-P-Ser/P-Thr antibody, indicating that it is present in the promastigote nucleus in a phosphorylated state (lane 1). A mock immunoprecipitation using IgG irrelevant to Lepp 12 was used as negative control (lane 3). Interestingly, in the total promastigote lysate (lane 2), the antibodies revealed an impressive variety of proteins of molecular weight ranging

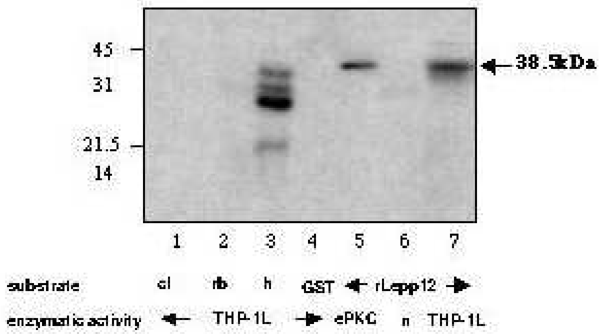


**Figure 9**
Enzymatic activity present in THP-1 cells phosphorylates recombinant protein Lepp12, in the presence of 10 microCi gamma-$P^{32}$ ATP in an *in vitro* assay. *E. coli* lysate (cl, lane 1), reaction buffer (rb, lane 2) and GST (lane 4) were used as negative substrate controls. Phosphorylation of histone mix (h, lane 3) by THP-1 lysate (THP-1L) and of GST-Lepp12 (rLepp12) by exogenous PKC (ePKC, lane 5) were performed as positive controls. n = no enzymatic activity added. Autoradiogram is shown after exposition time of 24 h.

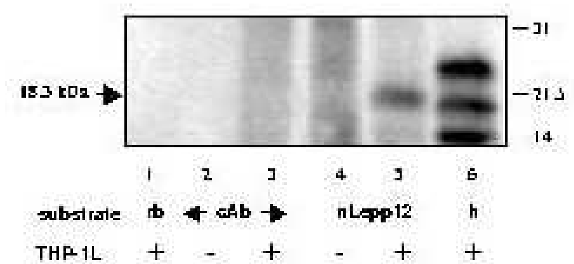


**Figure 10**
Enzymatic activity present in THP-1 cells phosphorylates natural protein Lepp12, in the presence of 10 microCi gamma-$P^{32}$ ATP in an *in vitro* assay. THP-1 lysate (THP-1L) was used as source of enzymatic activity. Natural Lepp12 (nLepp12, lanes 4, 5) was immunoprecipitated from the promastigote nuclear extracts (corresponding to $3 \times 10^9$ cells) using anti-Lepp12 IgG as described in FIG. 8. Control immunoprecipitation (cAb) was performed with irrelevant IgG (cAb, lanes 2, 3). Histone mix is also phosphorylated by an enzymatic activity present in THP-1 cells (h, lane 6). In this experiment, a phosphorylated monomeric form in the histone mix was detected, in contrast to the experiment depicted in fig. 9. Autoradiogram is shown after exposition time of 24 h.

between 14 kDa and 45 kDa which are phosphorylated in the cell on serine and/or threonine residues.

### *Phosphorylation of the recombinant and the natural Lepp12 by lysate of THP-1 cells*

The THP-1 myelomonocytic human cell line was used here since it provides a model of macrophages, the natural *Leishmania* host cells. We first examined the possibility of existence in these cells of kinase activity(ies) able to phosphorylate the recombinant and natural Lepp12. Figures 9 and 10 show that in both series of experiments, lysates prepared from THP-1 cell line were able to phosphorylate *in vitro* recombinant GST-Lepp12 (Fig. 9, lane 7) and natural immunoprecipitated Lepp12 (Fig. 10, lane 5), indicating the presence in this cell line of kinase(s) active on this leishmanial protein. The appropriate positive (Fig. 9, lanes 3, 5; Fig. 10, lane 6) and negative controls (Fig. 9, lanes 1, 2, 4, 6; Fig. 10, lanes 1–4) are described in the Figure legends.

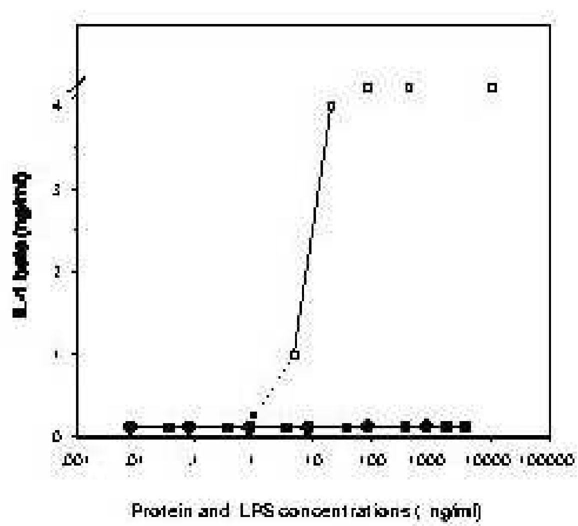


**Figure 11**
GST-Lepp12 and GST protein preparations used for transfection experiments (see FIGS. 12 and 13) are devoid of measurable endotoxin-like activity, as measured by IL-1beta synthesis by wild-type THP-1 cells. IL-1beta measured by sandwich ELISA in CHAPS lysate of THP-1 cells after 24 h culture of the cells ($5 \times 10^4$ cells per 200 microL) in the presence of GST-Lepp12 (closed diamonds) or GST (closed squares) was under the sensitivity threshold of the ELISA (10 pg/ml). LPS-induced IL-1beta (open squares) is shown as a positive control. It was above 4 ng/ml when LPS was used at concentrations over 10 ng/ml.

### *Effect of Lepp12 on IL-1beta production by THP-1 cells*

In order to investigate the influence of intracellular Lepp12 on macrophage activation and particularly on cytokine production by THP-1 cells, we generated cells transfected with the GST-Lepp12 and with GST using Chariot Transfection Kit. Prior to all the transfection experiments, all preparations of recombinant proteins were tested for the presence of bacterial contaminants, by measure of IL-1beta production by THP-1 cells cultured for 24 h in the presence of various dilutions of these preparations. Fig 11 shows that GST-Lepp12 and GST preparations were unable to induce detectable levels of IL-1beta from untransfected THP-1 cells. This lack of effect was observed even at protein concentrations above those selected in transfection experiments. This result indicates that both preparations were devoid of endotoxin-like activity as assessed by induction of IL-1beta synthesis, and therefore rules out an involvement of such an activity in the effects observed in GST or GST-Lepp12 transfected THP-1 cells. In the next series of experiments, the synthesis of IL-1beta was assayed in the THP-1 cells transfected with GST-Lepp12 or with GST, or untransfected, incubated in the absence and the presence of LPS (Fig. 12) or PMA (Fig. 13). The data show that IL-1beta basal production was undetectable in untransfected and GST-transfected cells whereas low but significant amounts (80–120 pg/ml) were reproducibly assayed from GST-Lepp12 transfected cells (Fig. 12 and 13). The LPS-induced IL-1beta levels were not significantly different in all cell types, whatever the effector concentrations. In contrast, the PMA stimulated monokine levels were higher in GST-Lepp12 transfected cells. The enhancing effect of Lepp12 on PMA-induced IL-1beta was more pronounced at low PMA concentrations (10-fold at 0.1 ng/ml versus 3.5-fold at 10 ng/ml). Taken together these results suggest that intracellular Lepp12 by itself or in synergy with PMA is specifically able to activate THP-1 cells to produce the pro-inflammatory cytokine IL-1beta.

## Discussion

In this paper we report the cloning and characterization of a novel *L. infantum* protein termed Lepp12. The 267 nucleotide long ORF was identified by screening a *L. infantum* cDNA library with an acute phase VL patient serum. The deduced 87 aminoacid sequence corresponds to a 11.6 kDa hydrophile, positively charged, protein with no homology with *L. infantum* protein sequences reported to date. Interestingly, Lepp12 exhibits 5 potential phosphorylation sites for protein kinase (PKC) and one N-glycosylation site. The fusion protein produced in *E. coli* and purified by glutathione-sepharose affinity chromatography showed by SDS-PAGE analysis one major band at the expected M.W. (38.5 kDa) and at least one smaller M.W. component at 34 kDa. As previously reported [30], partial proteolysis occurring during production in *E. coli* is likely to account for this phenomenon.

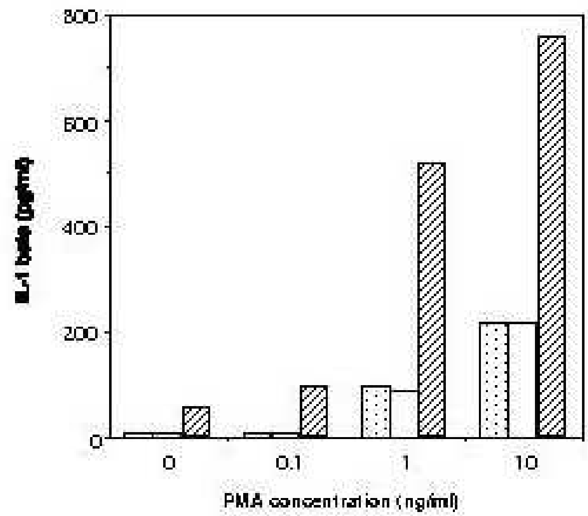


**Figure 13**
Intracellular Lepp12 activates THP-1 cells to produce IL-1beta and induces an enhancing effect on PMA-stimulated IL-1beta synthesis. IL-1beta was measured as described in FIG. 11 from THP-1 cells (7.5 × $10^4$ cells per 200 microL), untransfected (dotted bars) or GST-transfected (open bars) or GST-Lepp12-transfected (hatched bars), cultured in the absence or in the presence of PMA at indicated concentrations (in ng/ml). GST-Lepp12 protein and GST protein were transfected into the THP-1 cells using Chariot Transfection Kit as described in Methods section. One representative experiment out of three performed is shown

The purified fusion protein was used as capture antigen for evaluating the anti-Lepp12 antibody response in human with VL as well as in experimentally infected mouse and hamster. All patients tested were treated, following the clinical and parasitological diagnosis. Patients with obvious VL but not LST positive asymptomatic individuals showed at diagnosis anti-Lepp12 reactivity by ELISA or Western blotting (WB) which gradually declined following successful therapy resulting in clinical cure. Of note, VL patients serum samples recognized the low MW fusion protein compound in WB analysis thus supporting the Lepp12-like nature of this component. Conversely, anti-Lepp12 antibodies were undetectable by ELISA in experimentally infected mouse or hamster and attempts to prepare anti-Lepp12 antiserum in these animals by immunization with DNA encoding Lepp12 protein or purified GST-Lepp12 were either unsuccessful or resulted in

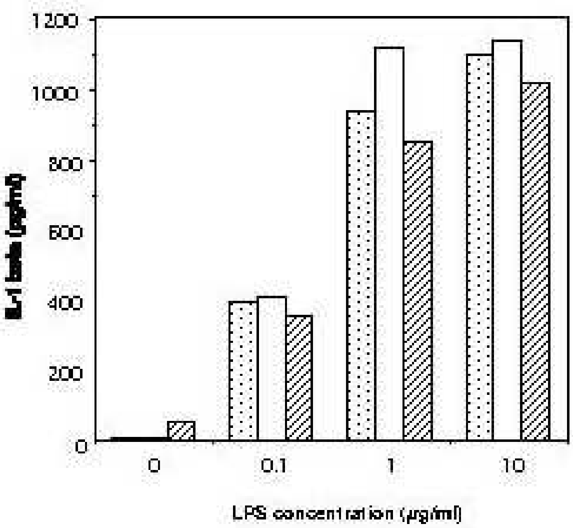


**Figure 12**
Intracellular Lepp12 activates THP-1 cells to produce IL-1beta and and has no effect on LPS-stimulated IL-1beta synthesis. IL-1beta was measured as described in FIG. 11 from THP-1 cells (7.5 × $10^4$ cells per 200 microL), untransfected (dotted bars) or GST-transfected (open bars) or GST-Lepp12-transfected (hatched bars), cultured in the absence or in the presence of LPS at indicated concentration (in microg/ml). GST-Lepp12 protein and GST protein were transfected into the THP-1 cells using Chariot Transfection Kit as described in Methods section. One representative experiment out of three performed is shown.

low titers immune sera, respectively. Consequently, immunopurified human anti-Lepp12, obtained by passage of *E. coli*-absorbed VL patient serum sample unto GST-Lepp12-coated latex column, were used as source of anti-Lepp12 antibody for the following experiments. This antibody preparation was shown to specifically detect by WB analysis the 38.5 kDa fusion protein as well as the low MW compound and was used to detect natural Lepp12 in crude promastigote preparation and in nuclear extract.

The species-specific humoral reactivity to Lepp12 deserves a comment. The fact that in infection of species other than human, the anti-Lepp12 antibody response was undetectable, while in human VL patients the response was detectable only at low serum dilutions, indicates that Lepp12 behaves as a weak immunogen, in contrast to papLe22. Moreover, the lack of a notable antibody response following extensive immunization of hamster, suggests existence of anti-Lepp12-repertoire or Lepp12-processing/presentation deficiencies in this and other animal laboratory species. Taken together, if one considers that Lepp12 is a weak immunogen, it is not surprising that only the most potent immune system can mount a detectable antibody response. Indeed, high responder character of human immune system to *Leishmania* proteins has been already emphasized, since anti-promastigote antibody responses measured in patients with VL exceeded by far those obtained with experimentally infected mouse or hamster or naturally infected dogs [31].

Natural endogenous Lepp12 appeared under two molecular entities migrating with apparent MW of 18.3 kDa and 14 kDa, the former being more represented. This discrepancy with the expected MW derived from the amino acid analysis can be explained by the occurrence in the promastigote Lepp12 of different glycosylation and/or phosphorylation states, the latter being known to modify the electrophoretic mobility of proteins [32]. Nevertheless, as reported earlier [33], the occurrence in promastigote fraction of two cross reactive entities with different MW cannot be totally excluded. In addition, there are three features indicating that tLepp12 could belong to the p14 and p18 nuclear fractions that we have previously reported [33]. First, the anti-Lepp12 antibodies recognize two bands of similar molecular weight to p14 and p18 fractions, second, p14 and p18 antigens share common epitopes and.third, Lepp12, p14 and p18 are all nuclear proteins. Finally, using RT-PCR Lepp12 mRNA was also detectable in amastigote, thus indicating that Lepp12 is likely to be present during parasite replication in host cell.

Amino acid sequence analysis of Lepp12 demonstrated 5 potential phosphorylation sites for PKC. In order to verify the functionality of these sites, several *in vitro* phosphorylation experiments were performed using as target either the fusion protein or immunocaptured natural Lepp12. GST-Lepp12 was phosphorylated *in vitro* by exogenous PKC and by PKC-like activities present in promastigote and in the myelomonocytic THP-1 cell line. These results indicate that at least one functional phosphorylation site is present on the recombinant Lepp12. In the same way, natural Lepp12, when immunoprecipitated, appeared to be also phosphorylated by both exogenous PKC and PKC-like activity present in THP-1 extracts. In promastigote, natural Lepp12, which was shown to localize in the nuclear fraction, appears under a constitutive phosphorylated state. Although there is not yet a direct proof, such as the natural protein sequencing or knock out experiments, of the identity of the Lepp12 ORF isolated from the library and the antigens recognized in the nuclear extracts, our data, brought to light in a logical sequence, provide a number of strongly converging indications for such identity. First, the recombinant Lepp12 (rLepp12) is phosphorylated in several *in vitro* assays. Second, a natural protein is recognized and localized in promastigote nuclear extract using antibodies affinity purified on rLepp12. Third, this natural protein is phosphorylated in the same vitro assays. Finally, this natural protein immunoprecipitated from promastigote nuclear extract is shown to be constitutively phosphorylated. There is an as yet unanswered question, namely whether and how does Lepp12 reach the host cell cytoplasm. At the present stage of our study we can hypothesize that Lepp12, being a protein strongly charged by positive residues, is able to cross membranes and to migrate in various compartments in a manner analogous to that of the TAT protein of HIV [34]. An additional, not exclusive, possibility is that Lepp12 presence in the host cell cytoplasm results from parasite destruction. A question, which seems related to this one, and to which there is no as yet a clear answer, is by which mechanisms the parasite nuclear proteins such as histones or papLe22 elicit strong immune responses in the vertebrate host. The concept of widely distributed antigens called panantigens with prominent immunogenicity addressed by Requena and collaborators [35] may answer this question.

Together our results indicate that the natural Lepp12 represents a substrate for PKC or other PKC-like activities or for phosphatase activities present in the promastigote and the host cell and therefore may interfere with signal transduction pathway involving PKC. This assumption was in part supported by the data obtained using transfection experiments. Indeed, unstimulated and PMA-stimulated GST-Lepp-12-transfected THP-1 cells produced markedly more IL-1beta than untransfected and GST-transfected controls whereas LPS-induced cytokine remained in all cases unchanged. These results indicate that in our *in vitro* model, Lepp12 interferes specifically with IL-1beta production dependent on PMA induced signaling pathway. The relevance of these findings lays in the crucial role played by IL-1beta as a main pro-inflammatory cytokine and as a main co-stimulatory factor of primary T-cell activation [36].

Several questions arising about Lepp12-transfected THP-1 cells are of relevance in order to better understand the role of Lepp12 in the host parasite interaction. First, does the presence of Lepp12 result in an increased transcription of the IL-1 gene and in this case, which are the IL-1 transcription factors that are modulated? Our observations showing that a *Leishmania* protein can be a putative macrophage activator are reinforced by a recent report [37] showing that *L. major* activates IL-1alpha gene transcription in macrophage cell line. Next, at what level of the signal transduction pathway is implicated the effect of Lepp12? Whether Lepp12 interferes with PKC directly or the downstream phosphorylation cascade leading to macrophage activation remains to be determined. Alternatively, Lepp12 under its phosphorylated form may inactivate phosphatases thus enhancing protein kinase activities involved in macrophage activation. These two hypotheses are of prime importance in the context of the parasite/host cell interaction. Indeed, invasion of macrophage by *L. infantum* or other *Leishmania* spp. was repeatedly reported to lead to a general deactivation of host cell with most genes being down regulated. This macrophage impairment following parasite entry includes innate and cell-mediated immune response such as phagocytosis [38], nitric oxide generation [39] and IL-12 production [40] and results in increased parasite survival inside the host cell [8,9]. A variety of mechanisms potentially contributing to macrophage deactivation during intracellular infection have been identified and among these, disruption of important target cell functions through interference with signal transduction is well documented. For example, infection with *L. donovani* selectively attenuates the IFNgamma-activated Jak-Stat1 pathway [17], reduces PMA-induced PKC activity [19] and impairs PKC-induced c-fos expression [41] or stimulates phosphotyrosine phosphatase SHP-1 [42]. On the contrary, *L. donovani* attachment was shown to stimulate PKC-mediated oxidative events in bone-marrow derived macrophages [43] and glycosylphosphatidylinositol of *L. mexicana* were found to activate PKC and protein tyrosine kinases in RAW 264 cells [44]. In this context Lepp12 appears, at least in our *in vitro* model, rather as a putative PKC enhancer. It also remains to be examined how our observations on the effect of Lepp12 on THP-1 activability can be translated to more physiological systems, in particular to Lepp12-transfected macrophages? Does Lepp12 impairs macrophage functions such as nitric oxide production? Finally, one should understand how an activation of one gene [37] or even of a series of genes by a protein can be integrated in the complex situation which occurs following parasite entry and which results in the global host cell deactivation. Indeed, it may be possible that during infection which involves numerous and complex amastigote antigens/host cell interactions, Lepp12 behaves quite differently and participates to the disruption of the protein kinase/protein phosphatase homeostatis leading to macrophage impairment.

## Methods

### *Cell cultures and cell preparations*

The promastigote form of *L. infantum* MON-1 (MHOM/FR/94/LPN101) was cultured in a complete RPMI medium at 25°C under usual conditions [33], except in some experiments indicated in the text where Fetal Calf Serum (FCS) was substituted by 0.1 % BSA. Early stationary phase promastigotes (5-day-old cultures) were used to carry out various cell preparations, unless indicated otherwise in figure legends, and were washed 3 times by sedimentation at 206 g for 5 min at 4°C in PBS containing 1 mM $NaVO_4$. For western blot analyses promastigotes were lysed in an electrophoresis buffer previously heated at 100°C for 10 min (100 mM Tris pH 6.8, 3 % SDS, 12.5 % glycerol). Promastigote lysates used to stimulate phosphorylation of recombinant Lepp12 were obtained as supernatants of centrifugation at 20,600 g for 15 min at 4°C after lysis of PBS-washed cells for 30 min at 4°C in water containing 1% NP40, 1 mM $NaVO_4$, 25 mM beta-glycerophosphate, 50 microM NaF, 2.5 mM NaPPi and 1 tablet (per 1 ml) of complete protease-inhibitor-cocktail (Roche, Meylan France). Nuclear extracts were prepared as follows: washed promastigote pellet was incubated for 10 min on ice in 0.5 ml of lysis buffer 1 (Hepes 10 mM, pH 7.5, $MgCl_2$ 1.5 mM, KCl 10 mM, DTT 0.5 mM, NP40 0.5 %, supplemented with proteases and phosphatases inhibitors as above), centrifuged (1,460 g, 10 min, 4°C), and the resulting pellet was incubated for 20 min on ice with 0.1 ml of lysis buffer 2 (Hepes 200 mM, pH 7.5, $MgCl_2$ 1.5 mM, KCl 840 mM, DTT 0.5 mM, glycerol 25%, EDTA 0.2 mM, supplemented with proteases ans phosphatases inhibitors). After centrifugation (15,500 g, 15 min, 4°C), the resulting supernatant was recovered. For immunoprecipitation experiments 0.1 ml of nuclear extract was first incubated with 23 microL of immunopurified anti-Lepp12 antibodies (or control, irrelevant human antibodies) for 5 h at 4°C under gentle agitation, then after adding 20 microL settled volume of mixed (1:4, v/v) protein A sepharose/sepharose 4B, incubation continued overnight as before. After centrifugation (20,600 g, 2 min, 4°C), 100 microL of 4X electrophoresis buffer supplemented with 16% betaME was added to the resulting pellet or the pellet was used in phosphorylation experiments (see below). The amastigote form used to prepare RNA was purified from hamster spleen [45]. Human monocytic cell line THP-1 was cultured in a complete RPMI medium and cell lysates used to stimulate phosphorylation of the recombinant and the natural Lepp12 were prepared as described above. The protein content in all cell preparations was measured using the Micro BCA Protein Assay Reagent kit, following supplier's (Pierce, Perbio, Bezons France) recommendations.

#### *Screening of cDNA libraries*

Two libraries of *L. infantum* promastigote cDNA (synthesized with oligodT primer or random hexaprimers) in lambda-gt11 bacteriophage were kindly provided by Dr. Carlos Alonso (Madrid). Approximately $10^5$ lambda-gt11 plaques were screened for each library, using an acute-phase patient serum as previously described [29].

#### *PCR amplifications, cloning and sequencing*

For cDNA synthesis, total RNA from $5.10^8$ *L. infantum* promastigotes and $5.10^7$ *L. infantum* amastigotes was extracted with 600 microL RLT lysis buffer (Quiagen, Courtaboeuf, France) following manufacturer's instructions and quantitated by spectrophotometry analysis. 2.5 μg RNA were reverse transcribed as previously described [46]. All PCR reactions were carried out using 0.2 mM deoxynucleoside, 1 microM of each primer and 0.014 U/microL of thermostable DNA polymerase (Q-biogene, Illkirch, France), in a final volume of 25 microL lambda gt11 inserts corresponding to the positive clones were amplified by PCR using specific phage primers and sequenced as previously described [29]. Specific primers were chosen for the clone termed 12 K and the total sequence of the coding region was obtained by RACE (Rapid Amplification of cDNA Ends)-PCR using high fidelity PWO polymerase (Boehringer Mannheim) and 5 microL *L. infantum* cDNA, as described previously (42). Briefly, the amplification of the 3' end of 12K cDNA was obtained with the specific primer F and the oligoT primer containing SalI site and its 5' end was obtained with the specific primer R and the "mini-exon" primer [47] with EcoRI site (underlined) 5'-TAG<u>GGATCC</u>AACTAAGCGTATATAAG TATCAGTTT-3'. After sequencing of 5' and 3' ends, the coding region corresponding to the clone 12K was amplified from *L. infantum* promastigote and amastigote cDNAs, using PWO polymerase and two specific primers F and R containing *EcoRI* and *SalI* sites, respectively. The clone 12K is termed thereafter Lepp12.

#### *Expression and purification of recombinant proteins*

PCR amplified coding region of Lepp12 (Lepp12-ORF) and pGEX-6P-1 vector (Amersham Pharmacia Biotech, Orsay, France) were digested with an excess of EcoRI and SalI (Biolabs Ozyme, Saint Quentin, France) restriction enzymes. The ligation between pGEX-6P-1 and Lepp12-ORF and the expression of the fusion protein with glutathione S-transferase (GST) in *E. coli* BL21 were performed as previously described [29]. The purification of GST-Lepp12 was done essentially as recommended by the supplier (Bulk GST Purification Module, Pharmacia). Briefly, the recombinant bacteria were harvested, washed once in NaCl 0.9% by sedimentation at 2500 g for 15 min at 4°C, resuspended in 1:20 volume of PBS containing protease inhibitors (complete protease-inhibitor-cocktail, Roche) and lysed by two sonication cycles of 10 seconds. After solubilization with 1% Triton X-100, the fusion protein GST-Lepp12 was adsorbed to glutathione-Sepharose gel (50% in PBS) for 30 min. After incubation, in order to obtain material without bacterial contamination, 10 washes with 10 volumes of PBS were carried out and the fusion protein was then eluted either using reduced glutathione 20 mM (in Tris-HCl 50 mM buffer pH 8) or with SDS 0.1%. Purified material was analyzed by SDS-PAGE (14% polyacrylamide gel), after staining with Coomassie G-250 stain (Invitrogen). *E. coli* BL21 transformed with pGEX-6P-1 vector without Lepp12-ORF were treated similarly, and the resulting recombinant GST was purified in parallel. *E. coli* BL21 not transformed were also treated similarly. For phosphorylation experiments (see below), the fusion proteins GST-Lepp12 and GST were used in a form adsorbed on the glutathione-Sepharose gel and were maintained in Assay Dilution Buffer (20 mM MOPS, pH 7.2, 25 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiotreitol, 1 mM $CaCl_2$). *E. coli* BL21 proteins adsorbed non specifically on glutathione-Sepharose gel, were used as additional controls.

### *ELISA*

The time course of specific anti-Lepp12 IgG levels in VL patient sera was determined by a classical enzyme-linked immunosorbent assay (ELISA) procedure analogous to that described previously for antileishmanial antibody determination [48]. Briefly, GST-Lepp12, or control GST, was coated overnight at 1 microg/ml (50 microL), the sera were tested at 1:100 dilution, and revealed with anti-human IgG peroxidase-conjugate used at 1:2000 dilution. Sera from leishmanin skin test (LST) negative subjects were used controls and resulted in OD values below 0.1. Incubation steps were performed in 0.1 M phosphate buffer pH 7.2 containing 1% (wt/vol) skimmed dry milk, 0.12% (vol/vol) Triton X-100, 0.2% (vol/vol) chloroform, 0.02% Thimerosal, 100 microg phenol red/ml.

### *Production of immunopurified anti-Lepp12 antibody*

Anti-Lepp12 antibodies were immunopurified from sera of VL patients in acute phase of VL on a GST-Lepp12-coated column. Briefly, 40 microg of GST-Lepp12 were coated on 200 microL of (2 times PBS-washed) Latex (Styrene divinylbenzene, 90.7 micron, Sigma), by overnight incubation at 25°C. The Lepp12-coated latex beads were mixed with 1 ml of sephadex gel G-25 (Pharmacia, France) and poured into a 2 ml column. The column was extensively washed with PBS and 0,1 M HCl-Glycine buffer pH 2,6 containing 0.5 M NaCl. VL patient serum (2 ml), previously absorbed with 0.5 ml of *E. coli* lysate, was loaded into the column. The column was washed in PBS and bound antibodies were eluted with the HCl-glycine buffer. After neutralization with 1 M Tris solution, concentration against dry PEG 35000 and dialysis with PBS, the antibody solution was supplemented with 2 mg/ml BSA filtrated on 0.22 micron millipore membrane and stored at 4°C. Typically 20 microg of immunopurified antibody were obtained from 1 ml of serum and was stored at 40 microg/ml concentration.

### *Western blot analysis*

Approximately 5 microg of the recombinant protein (GST-Lepp12 or GST) purified from a bacterial culture as described above, or 40 microg of total leishmanial proteins [33], were loaded per well of SDS-14 % polyacrylamide gel (mini-protean II cell, ref Bio-Rad) and electrotransferred to nitrocellulose (minitransblot cell, Bio-Rad) as previously described [29,33]. Patient and control (from LST negative subjects) sera were used at 1:50 and 1:100 dilutions and peroxidase-conjugates directed against human immunoglobulin G (Sigma Illkrich, France) were used at 1:100 and 1:1000 dilution, respectively. To reveal immunopurified anti-Lepp12 antibody, prepared as described above and used at 1:8, peroxidase-conjugates directed against human IgG were used at 1:500. Enzymatic activity was revealed with 1.5 mM diaminobenzidine, 0.38 mM $CoCl_2$, 0.03 % $H_2O_2$ in PBS. Alternatively, 120 microg (in 30 microL) of leishmanial proteins were loaded per well of mini-gel and electrotransferred to nitrocellulose membrane, as above. After saturation (10 mM Tris-HCl pH 7.4, 3 % BSA (Sigma, Illkrich, France), 150 mM NaCl, 1 mM EDTA, 0.1 %, Tween, 0.5 % Gelatine) for 2 h at 4°C, nitrocellulose was incubated with 0.25 mg/ml of anti-P-Ser/P-Thr antibody (Cliniscience, Montrouge France) for 18 h at 4°C, washed 3 times (1 % TBS-NP40, 10 min), then saturated again. Anti-rabbit immunoglobulin (IgG) peroxidase conjugate (Dako, Trappes, France) was used at 1:10000 dilution (1 h in saturation buffer). Enzymatic activity was revealed with ECL kit (Enhance Chemiluminescence, Amersham Pharmacia Biotech, Orsay, France) as recommended by the supplier on a sensitive photographic MP-hyperfilm (Amersham, Orsay, France). The quality of the transfert on nitrocellulose was regularly checked and confirmed by gel staining with Coomassie G-250 stain (Invitrogen, Netherland) and nitrocellulose membrane staining with Amido-Black.

### **In vitro** *phosphorylations*

Experiments were carried out with recombinant GST-Lepp12 or immunoprecipitated native Lepp12 and histone mix from bovine calf thymus (Upstate Biotechnology, Euromedex France, reference number14-155) as positive control and GST and BL21 lysate as negative controls, in accordance with the protocol supplied in PKC assay kit (Upstate Biotechnology, Euromedex France). Briefly, different proteins (10 microg) were incubated for 10 min at 30°C in the presence of 10 microCi gamma-$P^{32}$ ATP (ICN) in Assay Dilution Buffer (ADB) with sonified (90 sec) protein kinase C lipid activator, and with 25 ng exogenous PKC (Upstate Biotechnology, Euromedex, Mundolsheim, France) or 15 microg of promastigote lysate or THP-1 lysate prepared as described above. The reaction was stopped by addition of electrophoresis buffer, and the samples were electrophorezed and electrotransferred to nitrocellulose membrane. Phosphorylated material was revealed by autoradiograpy as described above. Exposition times are indicated in figure legends.

### *Transfection of recombinant GST-Lepp12 into THP-1 cells*

The transfection of the recombinant GST-Lepp12 protein into THP-1 cells was carried out using Chariot Transfection Kit (Active Motive, Rixensart, Belgium) following the manufacturer's instruction manual. Briefly, 3 × $10^5$ cells were seed in 3 ml of complete medium per well of a 6-well plate and cultured in usual conditions. After 48 h of culture, 200 microL of Chariot-protein complex (see below) was overlaid on the pellet of twice-washed THP-1 cells, and 400 microL serum-free medium was added to the overlay to achieve the final transfection volume of 600 microL. After incubation at 37°C for one hour, 1 ml of complete growth medium was added to the cells and incubation was allowed to continue for 2 more hours. The transfected cells were then used for activation experiments. The Chariot-protein complex formation was achieved by incubation of 100 microL protein dilution (2 microg of protein (GST or GST-Lepp-12) in 100 microL of PBS) with the 100 microL Chariot dilution (6 microL of Chariot in 100 microL of sterile water) at room temperature for 30 minutes.

### *Cell activation and cytokine assays*

In order to assess the potential presence of endotoxin-like material in GST and GST-Lepp12 preparations selected for the transfection experiments, THP-1 cells were cultured at $2.5 \times 10^5$/ml in the presence of various concentrations of both proteins for 24 h at 37°C in standard conditions. Culture wells were extracted with 9 mM CHAPS detergent and assayed for IL-1beta production by sandwich ELISA as previously reported [49]. The threshold sensitivity of the ELISA was 10 pg/ml and the technique was shown to quantify equally well the mature secreted and the intracellular forms of the cytokine. GST-transfected, GST-Lepp12-transfected and untransfected THP-1 cells (at $3.75 \times 10^5$ cells/ml) were challenged with various concentrations of phorbol-12-myristate 13-acetate (PMA, 0.1–10 ng/ml) or LPS (0.1–10 microg/ml) or left unstimulated. Culture wells were extracted and IL-1beta was quantified as described above.

### *Nucleotide sequence accession number*

The Lepp12 cDNA sequence obtained in this study has been assigned GenBank accession number AF540954.

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